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Remarks:
This application was filed on 17 - 06 - 1998 as a divisional application to the application mentioned under INID code 62.

(54) **A process for the purification of phosphatidylserine**

(57) The invention relates to a process for the purification of phosphatidylserines (PS) by selective extraction of PS-containing phospholipid mixtures in diphasic systems of organic solvents and, eventually, by further crystallization.

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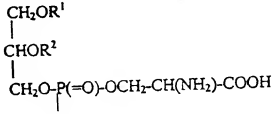
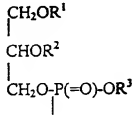
Description

[0001] The present invention relates to a process for the purification of phosphatidylserines of formula (I), hereinafter referred to as PS, by relying on the different partition coefficients in diphasic organic solvents.

[0002] The compounds which may be subjected to the process of the invention have the general formula (I) and are prepared starting preferentially from compounds (II), according to the procedure of European Patent Application n. 96119350.5-1212:



(I)



(II)

wherein R^1 and R^2, which are the same or different, are selected from a saturated, mono- or polyunsaturated C_{10}-C_{30} acyl group;
X = OH or OM wherein M is an alkali, alkaline-earth metal, ammonium or alkylammonium;
$R_3 = CH_2CH_2NH_2$ or $CH_2CH_2N^+(CH_3)_3$.

[0003] The importance of the compounds (I) is various, particularly in the preparation of pharmaceutical compositions for the therapy of involutive cerebral syndromes of different origin, such as vascular pathologies on atherosclerotic base or not and/or senile decline; for the preparation of liposomial formulations and more recently for dietetic compositions comprising natural lecithins, particularly soy lecithin enriched in phosphatidyl-L-serine, hereinafter referred to as PS(L), containing mostly polyunsaturated fatty acids as acyl residues.

[0004] The increasing demand for industrial amounts of PS(L) at a reasonable cost prompted the Applicant to carry out a thorough investigation to fulfill such a need.

[0005] Comfurius P. et al., Biochim. Biophys. Acta 488, 36 (1977) first discloses the production of an about 1/1 mixture of PS(L) and phosphatidic acid (PA), by reacting under pressure at 45°C and at pH 5.6, in a diphasic ethyl ether/water system, egg lecithin or synthetic phosphatidylcholines with L-serine in the presence of partially purified PLD enzyme (from cabbage).

[0006] PS(L) is then purified by chromatography on cellulose using a chloroform/methanol mixture as eluent. It is evident that this procedure is not suited to an industrial production both due to the use of ethyl ether and the low selectivity; furthermore the chromatographic procedure disclosed in this purification method is further ground against the industrial applicability of said methods for the use of chloroform in the eluent mixture, the low productivity and high cost.

[0007] The invention provides a method for the purification of the PS, relying on the different partition coefficients in diphasic organic solvent systems of phosphatides such as PS, PA, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and the corresponding lysophosphatides in the form of the corresponding salts, particularly the corresponding calcium salts in the diphasic heptane/methanol system.

[0008] It is possible to increase, in a 90% yield, the purity of a PS(L) obtained from Epikuron 200(R) from 88% to 95% thanks to its preferential ripartition in the heptane phase; similarly, the purity of a PS(L) obtained from Epikuron 135(R) was increased from 58% to about 80%. Finally, a further purification of PS can be obtained by crystallization from heptane/acetone in the form of the calcium salt and subsequent conversion into any other salt, according to conventional techniques.

[0009] The process of the invention can be conveniently applied for the purification of phosphatidyl-(L)-serines wherein R_1 and R_2 are acyl chains of palmitic, stearic, oleic, linoleic acids in similar proportions to that of soy lecithin or wherein R_1 and R_2 are acyl chains of palmitic, stearic, palmitoleic, oleic, linoleic, arachidonic acids in similar proportions to that of egg lecithin or wherein R_1 and R_2 are the same acyl chains in similar proportions to that of starting material (II).

[0010] The procedures reported in the following further exemplify the invention.

Example 1

Preparation of PS(L) starting from soy lecithin Epikuron 200

[0011] 20 g of Epikuron 200(R) (Lucas Meyer) and 100 ml of toluene are placed into a 1,000 ml reactor, under nitrogen, and the solution is concentrated under vacuum distilling about 80 ml of the solvent. Fresh toluene is added and the solution is concentrated again under reduced pressure. The procedure is repeated until reaching a content in ethanol or other C_1-C_4 alcohols, which are usually present in commercial lecithin, below 20 ppm. The residue is taken up into fresh toluene to a volume of 400 ml and added with 94.5 g of (L)-serine. The resulting suspension is added with the aqueous solution (300 ml) containing PLD from ATCC 55717, prepared according to the procedures of European Patent Application N. 96119350.5-1212 (Example 1) and having an enzymatic activity of 2 U/ml, added at 10°C with 3.34 g of calcium chloride, 4.08 g of sodium acetate trihydrate and about 3 g of glacial acetic acid to obtain a pH around 4.5. The resulting diphasic system is heated to a temperature of 25°± 2°C and kept under strong stirring for about 6h. The mixture is then filtered on decalite, which is further washed with 2x100 ml of toluene; the organic phase is separated from the aqueous phase containing the serine excess, and concentrated under reduced pressure to give a residue (22.3 g) which is taken up into 525 ml of n.heptane and 171 ml of methanol. The lower methanol phase is discarded whereas the higher one is further extracted with 220 ml of methanol. After separation, the higher phase is concentrated under vacuum to small volume, added, under stirring at -5°C, with 400 ml of acetone, filtered and dried under vacuum to give 15 g of PS(L) calcium salt with a 97% HPLC (PA content <3%).

Example 2

Preparation of PS(L) starting from soy lecithin Epikuron 135

[0012] 400 Kg of Epikuron 135(R) (Lucas Meyer), 3000 l of toluene, 100 l of water are placed into a 5,000 l stainless steel reactor, under nitrogen, and the mixture is concentrated under vacuum distilling at 45°C about 1,000 l of solvents. Another 6,000 l stainless steel reactor is loaded with 1,355 l of fermentation broth from ATCC 55717, containing about 3 KU/l of PLD, 22.7 kg of calcium chloride, 27.6 kg of sodium acetate trihydrate, and at 10°C 22 l of 80% acetic acid 625 kg of L-serine (final pH 4.2). The two solutions are combined and the resulting mixture is heated to and kept at 25°C with strong stirring for 8h. HPLC analysis shows a PS(L) content of about 75% of the total phospholipids. The mixture is then added with a suspension of 36 kg of decalite in 500 l of toluene and filtered, washing the filter with 400 l of toluene/water (3/1, V/V). The aqueous phase is then separated and treated to recover (L)-serine whereas the organic phase, after further filtration on decalite, is concentrated under vacuum to an about 440 kg residue, which is taken up into 5,000 l of acetone and stirred for about 6 h at room temperature. After cooling the mixture to 0°C, the product is filtered to give about 323 kg of PS(L) humid calcium salt (50%).

[0013] The product is further purified by treatment with 2,000 l of acetone and dried, to give about 273 kg of PS(L) calcium salt (58%).

[0014] A 20 g sample was purified by extraction with heptane/methanol, analogously to what described in example 1, to give 11,6 g of PS(L) calcium salt (80%).

Example 3

Preparation of PS(L) starting from egg lecithin.

[0015] 13 g of Ovothin 160(R) (60% PC; Lucas Meyer), 158 ml of toluene and 38 g of (L)-serine are placed into a 500 ml reactor, under nitrogen. The resulting suspension is added with the aqueous solution (300 ml) containing PLD from ATCC 55717, and having an enzymatic activity of 2 U/ml, added at 10°C with 1.4 g of calcium chloride, 1.7 g of sodium acetate trihydrate and glacial acetic acid necessary to obtain a pH of about 4.1. The resulting diphasic system is heated to a temperature of 25° ± 2°C and kept under strong stirring for about 6h. The mixture is then filtered on decalite which is further washed with 2x100 ml of toluene; the organic phase is separated from the aqueous phase, containing the serine excess, and concentrated under reduced pressure to give a residue which is taken up into 320 ml of n.heptane and 100 ml of methanol. The lower methanol phase is discarded whereas the higher one is diluted with 35 ml of heptane and further extracted with 95 ml of methanol. The higher phase is separated, concentrated under vacuum to small volume and added under stirring at -5°C with 250 ml of acetone to give, upon filtration and drying under vacuum, 7.3 g of PS(L) calcium salt with a 84% HPLC.

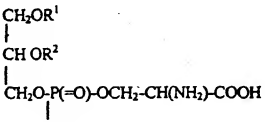
Example 4

Preparation of DLPS(L) starting from DLPC.

[0016] Repeating the procedure described in example 1, but using 20 g of L-α-dilinoneylphosphatidylcholine, referred to as DLPC, instead of 20 g of Epikuron 200(R), 15.1 g of L-a-dilinoneylphosphatidyl-L-serine, referred to as DLPS(L), as the calcium salt (96% HPLC purity), are obtained.

Claims

1. A process for the purification of the compounds of formula (I)



(I)

wherein:

R^1 and R^2, which are the same or different, are selected from saturated, mono- or polyunsaturated C_{10}-C_{30} acyl group;
X = OH or OM, wherein M is an alkali, alkaline-earth metal, ammonium or alkylammonium; by selective extraction of PS-containing phospholipid mixtures in diphasic systems of organic solvents.

2. A process according to claim 1, wherein phosphatidylserine is purified from the other phospholipids, particularly PC, PE, PA and the corresponding lysophospholipids, by selective extraction of the corresponding calcium salt from an heptane/methanol mixture.

3. A process for the further purification of phosphatidylserine obtained according to claim 2, wherein the phosphatidylserine is subjected to a crystallization from heptane/acetone, in the form of the calcium salt, and subsequent conversion into any other salt, according to conventional techniques.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 98 11 1111

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	US 5 084 215 A (KEARNS JOHN J ET AL) 28 January 1992 * claims 1,3 *	1-3	C07F9/10
A	COMFURIUS P ET AL: "THE ENZYMATIC SYNTHESIS OF PHOSPHATIDYLSERINE AND PURIFICATION BY CM-CELLULOSE COLUMN CHROMATOGRAPHY" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 488, no. 1, 20 July 1977, pages 36-42, XP000603420 * page 40, paragraph 2 *	1-3	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6) C07F

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	22 April 1999	Beslier, L

CATEGORY OF CITED DOCUMENTS

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 98 11 1111

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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22-04-1999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5084215 A	28-01-1992	US 4814111 A	21-03-1989
		US 4714571 A	22-12-1989

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82